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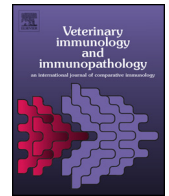
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Short communication

Functional characterisation of bovine TLR5 indicates species-specific recognition of flagellin[☆]

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ABSTRACT

Mammalian toll-like receptor 5 (TLR5) senses flagellin of several bacterial species and has been described to activate the innate immune system. To assess the role of bovine TLR5 (boTLR5) in the cattle system, we cloned and successfully expressed boTLR5 in human embryonic kidney (HEK) 293 cells, as indicated by quantitative PCR and confocal microscopy. However, in contrast to huTLR5-transfected cells, exposure of boTLR5-transfected cells to flagellin neither activated nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) nor CXCL8 production. Subsequent comparison of the flagellin response induced in human and bovine primary macrophages revealed that flagellin did not lead to phosphorylation of major signalling molecules. Furthermore, the CXCL8 and TNF α response of primary bovine macrophages stimulated with flagellin was very low compared to that observed in human primary macrophages. Our results indicate that cattle express a functional TLR5 albeit with different flagellin sensing qualities compared to human TLR5. However, boTLR5 seemed to play a different role in the bovine system compared to the human system in recognizing flagellin, and other potentially intracellular expressed receptors may play a more important role in the bovine system to detect flagellin.

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1. Introduction

Toll-like receptors (TLRs) comprise a family of transmembrane sensor proteins that sense the presence of

microbial associated molecular patterns (MAMPs) and induce innate immune responses (Akira and Takeda, 2004). With the advent of genomics, it has become evident that the TLR system is part of an ancient machinery that is evolutionary conserved with homologs present in insects, nematodes, plants, fish, mammals, and birds (Takeda, 2005). However, functional differences between related TLRs from different species exist and have been instrumental in deciphering TLR function, evolution, and susceptibility to infection (Jann et al., 2008; Keestra et al., 2008; Lizundia et al., 2008; Walsh et al., 2008). One family of that has recently gained more attention regarding its TLR repertoire due to its importance as a food animals and as potential reservoir for zoonotic pathogens are Bovidae.

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Analysis of the complete bovine genome indicated the presence of 10 different TLRs (Jungi et al., 2011) similar to other mammalian species. Ligand binding to TLR results in activation of the myeloid differentiation primary response gene (MyD) 88 signalling pathway and induction of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-regulated genes. For example, TLR5 is known to bind bacterial flagellin, the major subunit of the flagellum (Hayashi et al., 2001) and TLR5 is important in host defences against bacterial pathogens in several mammalian species. Indeed, TLR5 is functionally expressed on many types of epithelial cells, including those isolated from the intestinal, respiratory, and kidney/urogenital tracts and ocular surfaces. In the polarised epithelia derived from these organs TLR5 expression occurs at the basolateral surface. In contrast, TLR5 does not seem to be expressed at a functionally significant level in typical (i.e., non-mucosal) populations of mouse macrophages and dendritic cells, although it is expressed on such cells in humans (Means et al., 2003). Similarly, mRNA transcripts for boTLR5 were found in boMØ and bovine primary mammary epithelial cells, albeit at a low level (Porcherie et al., 2012; Werling et al., 2006).

Sequence alterations within the *tlr5* gene have been shown to have detrimental effects. A stop codon polymorphism in human *tlr5* present in approximately 25% of the human population is associated with an increased susceptibility to Legionnaire's disease (Hawn et al., 2003) but reduced inflammatory damage in cystic fibrosis (Blohmke et al., 2010). TLR5-deficient mice have an increased susceptibility to urinary tract infections yet lack pulmonary inflammatory responses (Andersen-Nissen et al., 2007a; Feuillet et al., 2006) and exhibit improved survival in experimental melioidosis (West et al., 2013). However, more recently the interaction of TLR5 with flagellin has also been shown to elicit anti-apoptotic and restitutive functions (Yu et al., 2006; Zeng et al., 2006). Intriguingly, *tlr5*^{-/-} mice are more resistant to systemic infection by *Salmonella enterica* serovar *typhimurium* (here referred to as *S. typhimurium*) and transport of the pathogen from the intestinal tract to the lymph nodes is impaired (Uematsu et al., 2006). Thus TLR5 exhibits pleiotropy and the functional status of TLR5 may be critical for the susceptibility, outcome, and the host range of infection.

Indeed, both flagellin (e.g., in *Escherichia coli*; Reid et al., 1999), and the extra-cellular domain (ECD) of TLR5 in primates (Wlasiuk et al., 2009) and other mammals (Areal et al., 2011) show evidence of adaptive positive selection. This suggests that interspecies competition between host and pathogen is possibly driving the co-evolution of pathogen and host. In support of this, species-specific single nucleotide variations in the TLR5 gene exist and a single nucleotide polymorphism (SNP) in the ECD of TLR5 in mice, chickens and humans is associated with a species-specific response to flagellin (Andersen-Nissen et al., 2007b; Keesstra et al., 2008).

The domestication of livestock by selection of desirable traits gave rise to the concept of breeds over 200 years ago (Taberlet et al., 2008). This formation of breeds by selective interbreeding offers a unique opportunity to examine an accelerated process of natural selection. We recently

investigated the evolution of the TLR5 gene in domestic livestock compared to other mammals using phylogenetic methods to identify species-specific and branch-specific evidence of positive selection, and identified known and novel SNPs in the coding region of TLR5 of sheep and cattle breeds (Smith et al., 2012). However, direct evidence that bovine (bo)TLR5 is functional and that the effects of flagellin are mediated via boTLR5 is lacking. The recent identification of additional (non-TLR) innate immune receptors that respond to bacterial flagellin (Franchi et al., 2006; Miao et al., 2006; Molofsky et al., 2006; Ren et al., 2006) warrants further investigation of the function and specificity of boTLR5.

2. Material and methods

2.1. Antibodies and ligands used

The antibody to the phosphorylated form of the p65 subunit of NF- κ B (Cell Signalling, UK) was diluted 1/1000 in 5% BSA/0.05% Tween20/1× PBS. Phosphorylation of the MAPK signalling family was analysed using the Anti-ACTIVE® MAPK Family Sampler (Promega, UK) of rabbit polyclonal Ab (pAb), which were diluted either 1:5000 (ERK1/2, JNK) or 1/2000 (p38) in 5% BSA/0.05% Tween20/1× PBS. Total p38 was analysed using p38 (N-20) rabbit pAb (Santa Cruz, UK) diluted 1/1000 in 5% non-fat-dried-milk/0.05% Tween20/1× PBS. Total β -actin was analysed using a murine monoclonal (m)Ab raised against β -actin (Millipore, Chemicon, UK) diluted 1/5000 in 5% non-fat-dried-milk/0.05% Tween20/1× PBS. Peroxidase labelled anti-mouse/rabbit Ab (Amersham ECL Western Blotting Analysis System, GE Healthcare), diluted 1:2000 in 5% non-fat-dried-milk/0.05% Tween20/1× PBS was used as secondary Ab.

TLR5 and TLR4 ligands were purchased from Invivo-gen, UK. Two different flagellin preparations derived from *S. typhimurium*, were compared: recombinant FlrC (rFlrC: *tlrI*-flrC; endotoxin-free) and purified flagellin (*tlrI*-stfla; 25 EU mg⁻¹). The TLR4 ligand was ultra-pure *E. coli* (O111:B4) LPS (*tlrI*-pelps; 10⁶ EU mg⁻¹).

2.2. Establishing of boTLR5-transfected HEK293 cells

HEK293 expressing huTLR5 cells were commercially available (Invivogen, UK) and maintained in HEK cell media supplemented with 10 μ g ml⁻¹ Blastcidin (Invivogen, UK). HEK293T cells, lacking expression of intrinsic TLR5 expression were maintained between 40% and 90% confluences, in DMEM supplemented with GlutaMAX (Gibco, UK), 10% FCS and 100 IU ml⁻¹/100 μ g ml⁻¹ penicillin-streptomycin. To clone boTLR5, total RNA was extracted from bovine MØ generated from a Holstein Friesian cow using the RNeasy Mini kit and SuperScript II reverse transcriptase as described by the manufacturer. Subsequently, boTLR5 was cloned by standard Easy-A High-Fidelity PCR using the following primers: Forward 5'-ATGGGAGACTGCCTTGACCT-3' and Reverse: 5'-CTAGGAGATGGTGGTTACATTTTGC-3'. The PCR reactions were run on a G-storm PCR machine (Gene Technology Ltd.) (95 °C for 2 min, followed by 35 cycles of 95 °C 1 min, 1 min primer annealing temperature and

72°C 1 min extension per 1 kbp, before a final extension at 72°C for a further 7 min). The resulting PCR product was run on a 1% agarose gel, and bands of the appropriate size were excised, extracted from the gel using the MiniElute Gel Extraction kit (Qiagen, UK), and concentration was measured using the NanoDrop (LabTech, UK). For sequencing, boTLR5 was cloned into pCR2.1 vector and propagated in OneShot TOP10 Competent Cells (Invitrogen, UK). Plasmid was extracted using a Plasmid Mini-prep kit (Sigma, UK), and sequence confirmed by BigDye Sequencing using a Big Dye Terminator v3.1 mix (Applied Biosystems, USA). The resulting boTLR5 sequences were assembled using CLC DNA Workbench 5.0.2 (CLC bio, UK) and deposited in GenBank (DQ335128). For subsequent expression as YFP-tagged receptor, EcoRV and XhoI restriction-sites were introduced onto the 5' and 3' ends of boTLR5, introducing a Kozak sequence at the start of TLR5 and removing the intrinsic stop codon (Forward: 5'-CGGCTTGATATCCACCATGGGAGACT-3'; Reverse: 5'-CGGCTTCTCGAGGGAGATGGTG-3'). After sequence confirmation, boTLR5 was cloned into the pcDNA3.1-YFP plasmid, and sequence again confirmed by primer walking sequencing (Mycosynth, Switzerland). Subsequently, HEK293T cells were transfected with boTLR5pcDNA3.1-YFP using the Amaxa Nucleofection system. After 24 h, the culture medium was replaced with selective medium supplemented with 1 µg ml⁻¹ Geneticin and cells maintained at 37°C (5% CO₂).

2.3. Confirmation and comparison of TLR5 expression levels

Levels of TLR5 mRNA transcription were analysed by qRT-PCRs using RNA extracted from transfected HEK cells, and was carried out in 48-well optical plates (Applied Biosystems, Life Technologies Corporation, Warrington, UK). 1 µg of reverse transcribed RNA was used as starting template, and each sample was measured in triplicate. Measurements were made using a StepOne qPCR machine (Applied Biosystems, Life Technologies Corporation, Warrington, UK) and StepOne software version 2.1 (Applied Biosystems, Life Technologies Corporation, Warrington, UK). PCR thermal cycling conditions for each amplicon comprised of one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Primers and probes for boTLR5 and huTLR5 were designed based on the publically available GenBank sequences DQ335128 and NM.003268, respectively. Their sequences were as followed: boTLR5 Forward: 5'-CCTGAATTCCTTCCACCAAG-3'; Reverse: 5'-GTCGCCAGGAAAAAGAACAG-3'; Probe: 5'-FAM-TTCATCATCTGACTGCACTGAGGGGA-3'TAMRA; and huTLR5 Forward: 5'-CTTCACATGGGTGCAGAATG-3'; Reverse: 5'-GATGGCATCTGGATATTGG-3'; Probe: 5'-FAM-GACTTTGTCCAGGAGAAAACCGCAT-3'TAMRA. The transcript copy number for a given gene was calculated by comparison with plasmid standard curves containing known copy numbers of target genes. Relative mRNA abundance values were then calculated according to the 'Relative Quantitation of Gene Expression Experimental Design and Analysis: Relative Standard Curve Method' (Applied Biosystems Technical Bulletin: 'Guide

to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR'). In addition to the assessment of mRNA expression, protein expression was monitored by flow cytometry. In the absence of antibodies detecting conserved residues for boTLR5 and huTLR5, expression levels or recombinant TLRs were compared based on expression of YFP using a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). Data were analysed using FlowJo (version 7.2.5; TreeStar, USA). In addition, boTLR5-transfected HEK293T cells were selected from untransfected/non-expressing cells by cell sorting using a FACSria Cell sorter (Becton Dickinson).

2.4. Dual luciferase assay

HEK cell-lines were seeded at 2×10^5 per ml of DMEM (without antibiotics), supplemented with 10% FCS, in a 24-well plate and incubated overnight (37°C, 5% CO₂). Dual luciferase assay was performed as described recently (Lizundia et al., 2008). Briefly, cells were transiently transfected for 24 h with 2 ng µl⁻¹ luciferase-tagged NF-κB (NF-κB-luc) plasmid and 0.4 ng µl⁻¹ Renilla (pRL-TK) plasmid using Lipofectamine (Invitrogen, UK) according with the manufacturer's recommended guide-lines. Transfected HEK cell-lines were then exposed to: 0.1 µg ml⁻¹ rFliC, 1 µg ml⁻¹ purified flagellin or control medium, over a 48 h time-course. At each time-point supernatants were harvested and stored at -20°C. In addition, protein was extracted from the cells using 100 µl 1× Passive lysis buffer (Dual-Luciferase Reporter Assay System, Promega). NF-κB-luc and Renilla expression were analysed on a MicroLumat Plus LB96V luminometer (Berthold Technologies) and luciferase values were recorded using WinGlow software (v.1.25.00003, Bethold Technologies).

2.5. Generation of primary macrophages

All procedures were in accordance with Home Office License regulations and RVC Ethics Committee approval. Blood was collected by venous puncture from five healthy Holstein Friesian cows that tested negative for BVDV antibodies and viral RNA, IBR, and *Mycobacterium bovis* were housed at Bolton Park Farm. Bovine peripheral blood mononuclear cells (PBMC) isolated by an adapted Ficoll-Histopaque procedure were used for flow cytometry, or for the generation of monocyte-derived MØ (Jungi et al., 1996b). For generation of MØ, PBMC were sealed in Teflon bags (10–20 ml, 4×10^6 PBMC per ml) as described previously (Jungi et al., 1996b) and cultured for 6–8 days at 37°C in a humidified atmosphere of 5% CO₂. The medium was RPMI-1640 containing 10 mM HEPES (pH 7.4), 100 IU ml⁻¹ penicillin/100 µg ml⁻¹ streptomycin, 1% v/v non-essential amino acids for minimal essential medium (MEM; Invitrogen, Basel, Switzerland), 0.4% v/v vitamin solution for MEM (Invitrogen), 2 µg ml⁻¹ glutamine (Invitrogen), 40 µg ml⁻¹ folic acid, 1 mM sodium pyruvate (Invitrogen), 2.5 µg ml⁻¹ amphotericin B (Invitrogen) and 15% heat-inactivated foetal calf serum (FCS; Invitrogen). During this time, monocytes had matured to non-activated MØ, which optimally responded to LPS (*E. coli* O11:B4; 100 ng ml⁻¹, Invivogen) and Gram-negative organisms

by NO generation and TNF α production (Jungi et al., 1996b). From the cell mixture of variable composition, M ϕ were purified by selective adherence to microtiter plate wells for 3 h. After washing, the level of T-cell contamination was estimated to be 1–2%, based on immunocytochemical analysis (unpublished observation), with a viability >98%. For generation of human M ϕ , PBMC were isolated from human blood as described above using Histopaque-1077 ($d = 1.077 \text{ g ml}^{-1}$, Sigma, UK).

2.6. Activation of macrophages

M ϕ were seeded at a density of 8×10^4 cells per 100 μl boM ϕ culture medium supplemented with 2% FCS, in 96-well flat-bottom plates (Greiner bio-one, CELLSTAR, Scientific Laboratory Supplies Ltd, UK) and allowed to adhere for 16 h (37 °C, 5% CO $_2$). TLR ligand doses were optimised initially by analysing the level of CXCL8 in the supernatant, after exposing M ϕ over a 24–48 h time-course with 10-fold dilutions ($1 \mu\text{g ml}^{-1}$ to $0.01 \mu\text{g ml}^{-1}$) of rFliC, purified flagellin and LPS. Supernatants were stored at –20 °C before analysing protein.

2.7. ELISA for CXCL8 and TNF α

To assess whether potential effects seen at the level of NF- κ B activity may also affect other parameters, CXCL8 and TNF α production was analysed in supernatants harvested from the above described experiments using either a commercially available ELISA according to the manufacturer's instructions (Quantikine Human CXCL8/IL-8 ELISA; R&D Systems, Abingdon, UK) as described recently (Lizundia et al., 2008), or a bovine specific TNF α ELISA (Kwong et al., 2010).

2.8. Western blotting

Flagellin preparations were diluted to 20 ng μl^{-1} in Laemmli buffer. Protein was extracted from cells at 90% confluence seeded in either 24-well or 6-well culture plates by 10 min incubation with 100 μl and 200 μl lysis buffer (M-PER $^{\text{®}}$ Mammalian Protein Extraction Reagent (Thermo Scientific, UK) containing $1 \times$ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, UK)), respectively. Protein extracts were agitated in the plate and spun in 0.5 ml Eppendorfs for 15 min at $13,000 \times g$. The liquid lysate was carefully removed and mixed with $5 \times$ Laemmli buffer to make a $1 \times$ Laemmli buffer concentration. All protein extracts were boiled at 95 °C for 3 min before loading on a 10% Ready Gel (BioRad, UK) and separated using the MiniPROTEAN 3 Cell system (BioRad, UK) at 30 mA for 1 h. Separated proteins were transferred onto a Hybond-ECL membrane (Amersham, GE Healthcare UK Ltd., Little Chalfont, Bucks, UK) using the MiniTrans-Blot Electrophoretic Transfer Cell system (BioRad, UK) at 100 V for 1 h. Membranes were rinsed gently in deionised water and blocked for 1 h at RT using the primary Ab dilution-reagent, followed by overnight incubation with the primary antibody at RT. Membranes were rinsed $2 \times$ and washed $3 \times$ for 10 min using 0.05% Tween/1 \times PBS and incubated with secondary Ab for 1.5 h. Membranes were washed as described

and incubated with detection reagents (Amersham ECL Western Blotting Analysis System) prior to film exposure.

2.9. Statistical analysis

Each transfection was performed in triplicate and each experiment was repeated as indicated. Data are presented as fold increase related to untransfected cells. For CXCL8 and TNF α production, the absolute values are given. All ELISA tests were performed in duplicate, and experiments were performed at least twice. Statistical values are shown for pooled data obtained from at least two experiments. Initially, data were assessed for normal distribution using D'Agostino and Pearsons omnibus normality test. Thereafter, an unpaired *t*-test with Welch's correction was performed for data shown in Fig. 4.

3. Results and discussion

The main aim of the present experiment was to further elucidate the functional role of flagellin-TLR5 interaction in the bovine system.

Initially, gene reporter assays were used to characterise the response of boTLR5-HEK293, huTLR5-HEK293 and untransfected HEK293 to either rFliC, purified flagellin or control medium over a 48 h time course. Both flagellin preparations induced a strong and rapid NF- κ B activation and CXCL8 production in huTLR5-HEK293 cells (Fig. 1A and B), although purified flagellin induced a stronger and faster response compared to rFliC. In contrast, stimulation of boTLR5-HEK293 (Fig. 1C and D) and untransfected HEK293 cells (Fig. 1E and F) with either flagellin preparation did not result in substantial NF- κ B activation or CXCL8 production. The differences in the responses seen in huTLR5-HEK cells to the two flagellin preparations could be explained by the varying concentrations used as well as potential differences in structure or composition of flagellin preparations. Indeed, it has been confirmed previously by western blot that recombinant FliC exhibited a greater molecular weight compared to that of purified flagellin, which was unique to FliC and was not observed for FljB (Metcalfe et al., 2010). FliC has been reported to confer greater pro-inflammatory activity compared to FljB whereas both were shown to induce similar responses in HEK cells (Simon and Samuel, 2007). Also, the different compositions of rFliC and purified flagellin could affect the efficiency of binding to TLR5 or other receptors, as has been described recently for *Pseudomonas aeruginosa* flagellin (Vermin et al., 2005). With regards to the differences seen between huTLR5- and boTLR5-transfected HEK cells, it is unlikely that these observations were due to variations in gene expression. qPCR analysis for TLR5 transcripts confirmed similar levels of transcripts for both TLR5 molecules (Fig. 1G), and boTLR5 was clearly expressed as protein in HEK293 cells (Fig. 1H), as confirmed by confocal microscopy.

Recently we were able to demonstrate sites of positive selection in boTLR5 (Smith et al., 2010). Indeed, when the ratio of synonymous and non-synonymous single nucleotide polymorphisms between lineages or clades were compared, the artiodactyl lineage and the individual porcine, ovine and bovine lineages comprising this

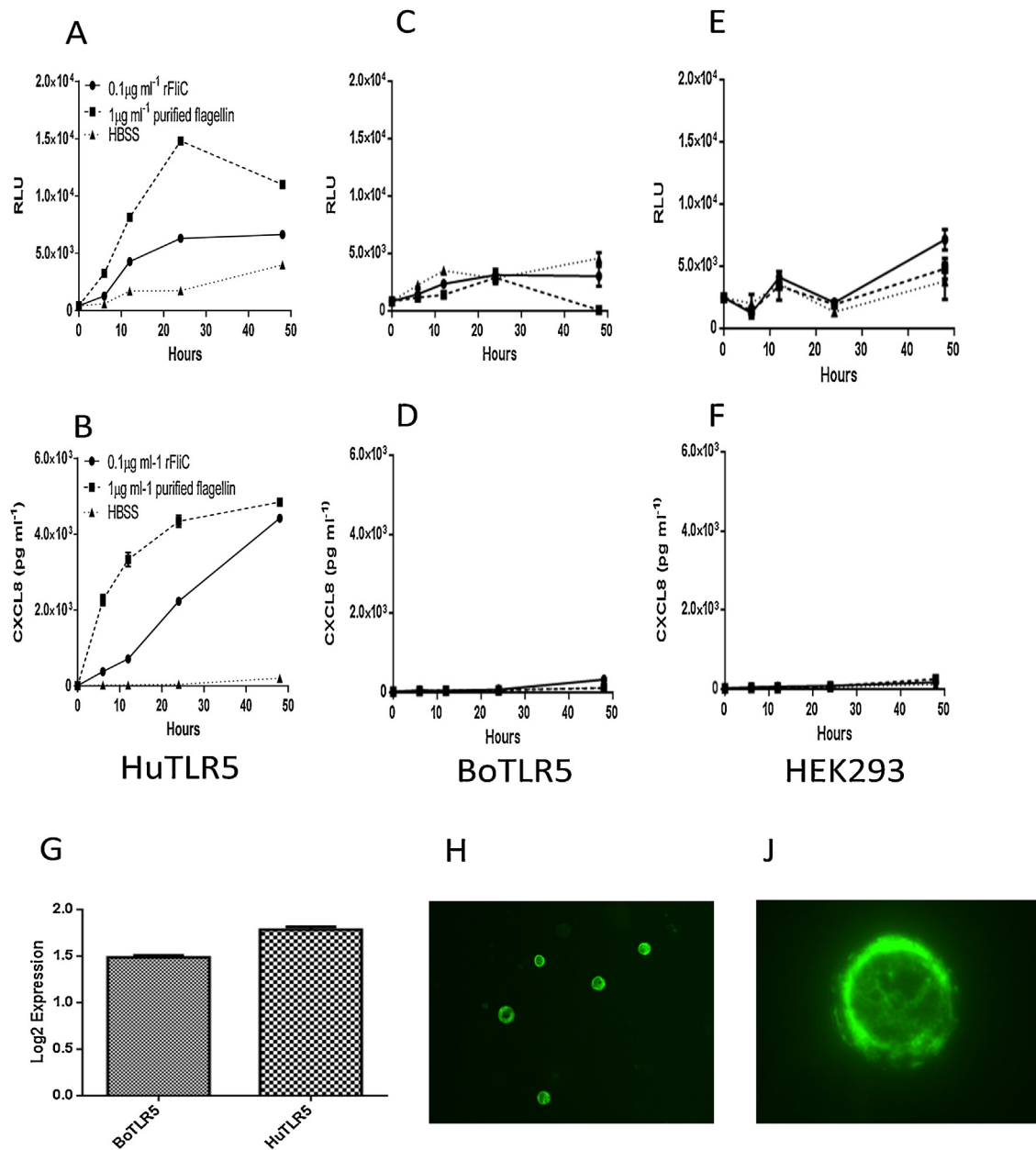


Fig. 1. Transfecting HEK293 cells with human, but not bovine TLR5 stimulates a NF-κB and CXCL8 response. HEK293 cells were transiently transfected with NF-κB-luciferase/Renilla and challenged over 48 h as described. Protein extracts were analysed for NF-κB-luciferase expression (A, C, E) and supernatants were analysed for CXCL8 (B, D, F) as described. (G) TLR5 mRNA expression levels in HEK293 cells transfected with either bovine or human TLR5; (H–J) expression of BoTLR5–YFP in HEK293 cells taken at a magnification of 20× (H) and 40×, respectively using a fluorescent microscope with a filter of 488 nm wavelength. A representative set of data from 3 individual experiments is shown.

clade exhibited significant evidence of adaptive evolution. Positive selective pressure on genes is symptomatic of functional adaptations acquired during the evolution of species and can promote species functional diversification (Vamathevan et al., 2008). We postulated that adaptive evolution observed in TLR5 of domestic livestock is a result of the breeding process, and that selective breeding indirectly drives changes in host–pathogen interactions. Indeed, the majority of the positively-selected sites are positioned in the ECD of TLR5—the part of the

protein involved in flagellin recognition. These differences have been clearly mapped (Smith et al., 2010) and it is evident that variability within this region promotes species-specific ligand recognition (Andersen-Nissen et al., 2007b). Evidence of increased positive selection within the extracellular domain supports the hypothesis that competition with flagellated bacterial pathogens is driving adaptation in specific host TLR5 ECD and more precisely in the flagellin-binding region. We now postulate that these host-specific adaptations in TLRs contribute to colonisation

and infection by microbiota and pathogens and could be crucial in determining outcome of infection by the same organism in different host species.

TLR5-flagellin interaction has recently been mapped at the single amino acid level (Andersen-Nissen et al., 2007b; Lu and Sun, 2012; Yoon et al., 2012) suggesting that the exchange of a few amino acids within either TLR5 or flagellin could alter the TLR5 responses in a species-specific or strain-specific fashion which in turn, may influence the host range and their susceptibility to infection. Such amino acid exchanges could explain some of the biological differences in the response of different species to flagellin. Chicken TLR5 has been shown to recognise different flagellin forms compared to human or murine TLR5 (Keestra et al., 2008). These differences are partially based on flagellin amino acid differences, and the exchange of these amino acids between different flagellins alters their interaction with TLR5 from different species (Keestra et al., 2008; Murthy et al., 2004).

To assess whether the lack of NF- κ B activation in response to flagellin in boTLR5 transfected HEK293 cells was also present in primary cells, boM ϕ were stimulated with rFliC or purified flagellin as well as LPS. After a 30 min exposure to ligands cells were lysed and the protein extracts analysed for the presence of phosphorylated isoforms of signalling components (phosphorylated p65 subunit of NF- κ B, phosphorylated kinases p38, ERK1/2, and JNK). Whereas LPS induced phosphorylation of all the molecules analysed, neither rFliC nor purified flagellin induced strong phosphorylation of these signalling molecules (Fig. 2A). In fact, when the relative intensity of bands on western blots were analysed in comparison to β -actin, a 4-fold increase in phosphorylation was seen with LPS (Fig. 2B), whereas neither rFliC nor purified flagellin induced phosphorylation above the values obtained with the negative control (Fig. 2B).

As it was suggested that flagellin may induce CXCL8 production via stimulation of the p38 kinase, and not exclusively via NF- κ B activation (Jungi et al., 1996a), we next compared the response of boM ϕ and huM ϕ to the same batches of $1 \mu\text{g ml}^{-1}$ purified flagellin, $1 \mu\text{g ml}^{-1}$ LPS or negative medium. Protein extracts were analysed for total p38 (Fig. 3A) and phosphorylated p38 (Fig. 3B) by Western blotting. Total p38 remained at a constant level (Fig. 3A) under all conditions tested. As before, LPS induced significantly more p38 phosphorylation in boM ϕ compared to purified flagellin, when compared to β -actin. In huM ϕ , p38 phosphorylation seemed to be already consistently higher in the unstimulated stage, and stimulation of huM ϕ only led to a small increase in p38 phosphorylation (Fig. 3B).

As boM ϕ seem to possess the ability to respond to flagellin (Metcalfe et al., 2010), we next assessed whether the apparent lack in flagellin responsiveness was specific to the HEK293 system or a general phenomenon of boTLR5 by comparing the CXCL8 and TNF α response of primary huM ϕ and boM ϕ to rFliC over a 24 h time course, (Fig. 4). For both cytokines tested the response to rFliC ($1 \mu\text{g ml}^{-1}$) was stronger in huM ϕ compared to that seen in boM ϕ (Fig. 4A and B), despite the fact that an rFliC dose-dependent response was seen in boM ϕ for TNF α production (Fig. 4C). This was similar to the result described

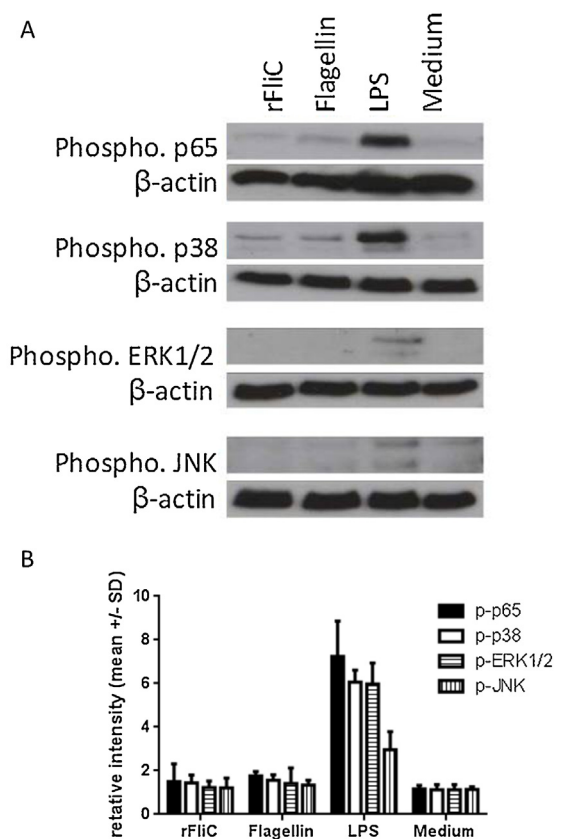


Fig. 2. LPS, but not flagellin stimulates phosphorylation of main signalling molecules in bovine primary macrophages. BoM ϕ were generated as described and stimulated for 30 min with either $0.1 \mu\text{g ml}^{-1}$ recombinant (r)FliC, $1 \mu\text{g ml}^{-1}$ purified flagellin (flagellin) or $1 \mu\text{g ml}^{-1}$ LPS. Protein extracts were probed by western blotting for phosphorylated isoforms of p65, p38, ERK1/2 or JNK. Band intensities were measured in ImageJ and the relative intensity was calculated after normalisation to β -actin. A representative set of western blots is shown, relative intensities represents mean \pm SEM of 4 individual animals.

previously for CXCL8 production (Metcalfe et al., 2010) and indicated that the cells have the ability to detect and respond to flagellin. Several factors may contribute to the differences seen between the HEK cell system and the primary M ϕ . One factor could be the purity of the ligands used. As expected, LPS contained the highest endotoxin level (tlr1-pelps – 10^6 EU mg^{-1}) whilst low contaminating levels of LPS were present in rFliC and purified flagellin (tlr1-stfla – 25 EU mg^{-1}). Therefore, in addition to potential structural alterations between the flagellins as mentioned previously, the CXCL8 and TNF α responses could be caused by LPS-TLR4 interactions occurring on primary M ϕ , rather than only the interaction of flagellin with TLR5. Whereas the alterations in the responses to the two flagellin preparations can be explained relatively easily, the differences seen between the two species are somewhat puzzling, but not unique. Indeed, our present data are similar to data derived from equine M ϕ (Kwon et al., 2011), indicating a lack of TLR5 responsiveness to flagellin in this species. In addition, as stated above, huM ϕ seem to clearly express TLR5 (Means et al., 2003), whereas only low level of TLR5 mRNA

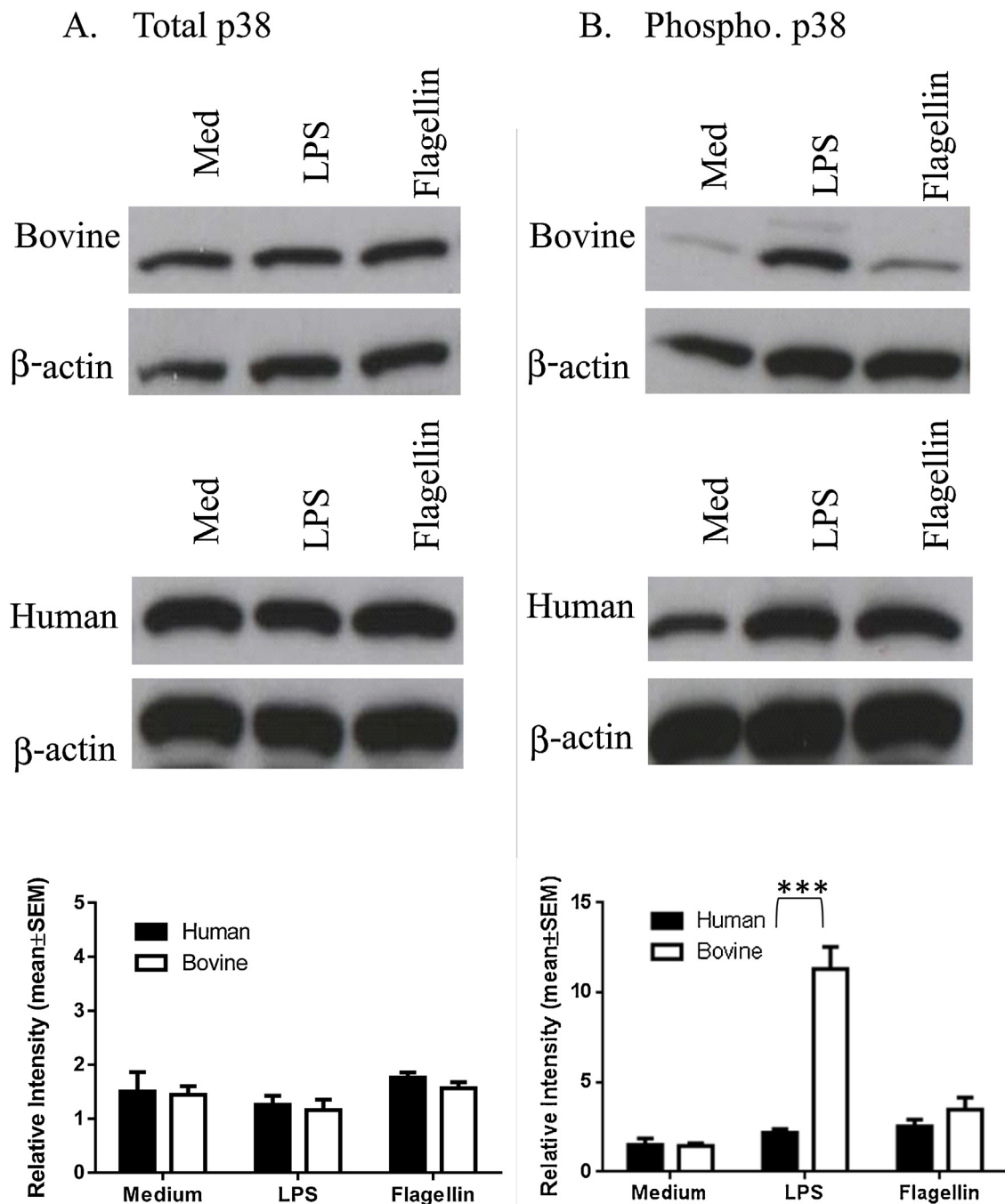


Fig. 3. Purified flagellin and LPS induced different levels of p38 phosphorylation in bovine and human primary macrophages. BoMØ and huMØ were generated as described and stimulated for 30 min with either $1 \mu\text{g ml}^{-1}$ purified flagellin, $1 \mu\text{g ml}^{-1}$ LPS or left in medium. Protein extracts were probed by western blotting for total p38 (A) and phosphorylated p38 (B), in comparison to β -actin. Band intensities were measured in ImageJ and the relative intensity was calculated after normalisation to β -actin. A representative set of western blots is shown, relative intensities represents mean \pm SEM of 3 individual animals. (***) $p < 0.001$.

transcripts were detected in boMØ (Werling et al., 2006). If these expression differences are truly reflected on the cell surface, it is possible that flagellin detection in huMØ occurs via surface expressed TLR5, resulting in the downstream activation of NF- κ B. In contrast, flagellin detection in boMØ may occur via intracellular flagellin receptors.

Indeed, mammals have evolved at least two distinct pathways of recognizing flagellin monomers. Specifically, extracellular flagellin is recognised through toll-like receptor 5 (TLR5), whereas intracellular flagellin is recognised through a pathway apparently involving both interleukin-converting enzyme protease-activating factor (Ipa) and

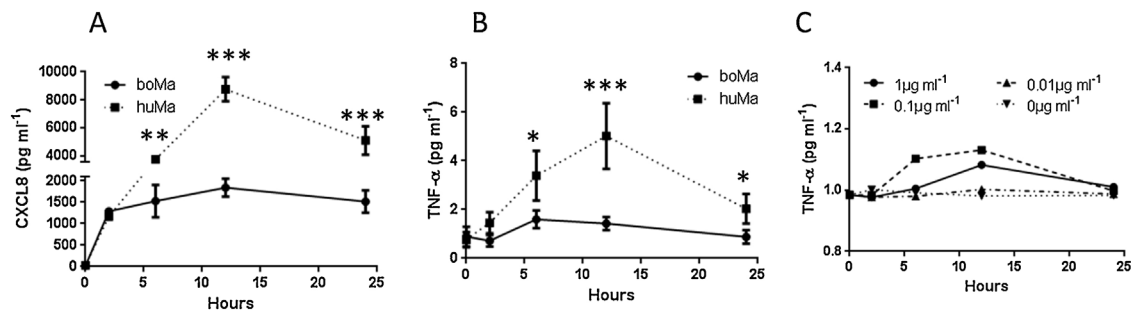


Fig. 4. Stimulation of human and bovine primary macrophages flagellin induces CXCL-8 and TNF α production. HuM ϕ and boM ϕ were generated as described and stimulated for the time points indicated with rFLiC, and CXCL8 and TNF α concentrations were analysed by ELISA as described (A and B). In addition, the TNF α response to rFLiC was analysed over the time period indicated for different rFLiC concentration in boM ϕ . Data are presented as mean \pm SEM for bovine samples ($n=5$) and human samples ($n=2$) (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Nod-like receptor apoptosis-inhibitory protein-5 (Naip5) (Miao et al., 2007). Whereas earlier data suggested that the interaction of Ipaf with flagellin has to occur by cytosolic flagellin being injected via a type-III secretion system (Lightfield et al., 2008), more recent evidence suggest that Ipaf can also be activated in response to purified flagellin monomers (Sanders et al., 2009).

While usage of different pathways could potentially explain the differences seen between human and bovine primary cells, this does not explain the differences seen using TLR5-transfected HEK cells. Two potential hypotheses may help to explain this. The first one would consider the copious quantities of Gram-negative flagellated bacteria present within the GIT system as mucosal commensals. In this scenario, boTLR5 may work more like a “buffering” PRR, similar to the interaction of LPS with LPS-binding protein and/or MD2, to avoid an overshooting activation of the innate immune response to small amounts of flagellin. However, using higher concentrations of the *S. typhimurium* flagellin did not induce a response in boTLR5 transfected HEK cells. The second hypothesis considers that boTLR5 only gets activated when forming heterodimers with TLR4, similar to that described in the murine system (Mizel et al., 2003). However, such a system would be difficult to set up experimentally, as it would involve the transfection (and potential selection) of HEK cells with at least four different plasmids (TLR5, TLR4, CD14, MD2) to obtain full activation. Another potential explanation could be that boTLR5 in Holstein-Friesian cows represents a loss-of-function mutation. Such a mutation has been described for huTLR5 in a high population frequency (up to 23%) (Barreiro et al., 2009; Gewirtz et al., 2006; Hawn et al., 2003), potentially supporting the notion of a largely redundant role of TLR5, and suggesting that other accessory mechanisms of pathogen recognition, either via Ipaf/Naip3 or location specific expression of TLR5 may provide sufficient protection against infection. Indeed, initial analysis for species-specific amino acid substitutions showed clear differences between the ruminant TLR5 sequence and human TLR5 sequences (Smith et al., 2012; Offord and Werling, unpublished observation).

The data presented in the current study potentially challenge our view of TLR5 as a PRR inducing a pro-inflammatory response via NF- κ B in the bovine system. As flagellin is currently used in clinical trials as a vaccine

adjuvant in the human system our data, if corroborated, may impact on the use of flagellin as vaccine adjuvants in the bovine system. Thus, further studies are needed to elucidate the role of TLR5-flagellin interaction in the bovine system.

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